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Purification and Characterization of Lipase from Moderate halophiles isolated from Excreta of Wild Ass (*Equus hemionus khur*)

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ABSTRACT:
Twenty-four moderate halophiles were isolated from excreta of wild ass on medium containing 10% NaCl (w/v), designated as Mk-1 to Mk-24. Three best lipase producers were judged on the basis of zone ratio on solid media were selected for further study. Extracellular lipase purification was achieved by ammonium sulfate fractionation after growing organisms in liquid media containing tributyrin as carbon source. Maximum purification of lipase from Mk-4, Mk-18 and Mk-23 was obtained in 60%-80% saturation fraction. Extracellular lipases were active maximally in acidic pH (5-6) and mesophilic temperature (30-40˚C). Additionally, lipases denatured at higher temperature and by chemicals like NaF, EDTA, SDS and Urea. Increase in lipase production up to 3.4 U/ml was obtained after UV mutagenesis in Mk-23.

Key words: Moderate halophiles, wild ass, Halophilic lipase, Enzyme characterization

INTRODUCTION:

The wild ass sanctuary is located in the little rann of Kutch of the Gujarat State in India. It covers an area of 4954 km². The sanctuary is named after a sub species of wild ass (*Equus hemionus khur*), the last population of which it harbours. The present saline desert of the little rann (saline desert-cum-seasonal wetland) of Kutch is believed to have been shallow sea.

Organisms that can tolerate harsh conditions like extreme pH, temperature, salt etc. are categorized as extremophiles. Extremophilic diversity is widely studied around the world, since they produce enzymes which are able to work under extreme conditions and can be potentially used for many biotechnological and industrial applications [1, 2]. Halophiles are a group of extremophiles that are able to tolerate high salt concentrations. Halophiles can produce salt and heat tolerant extracellular hydrolytic enzymes like amylases, proteases, lipases, pullulanases, xylanases, DNases, etc. which have applications in food industries, detergent additives, paper industries, pharmaceutical industries, chemical industries etc. [3, 4, 5].

Lipolytic enzymes (EC 3.1.1.3) are hydrolases group enzymes with catalytic activity on carboxylic ester. Lipases are widely used in fat/ oil processing, detergent formulation, paper-pulp industries, food industries, cosmetics and pharmaceuticals [6, 7], polyurethane [8] and biodegradation of fatty acid containing waste [9]. Present day, lipase research is focused on kinetics studies, determination of 3 D structure and genetic modification in lipase producing genes [10].

MATERIALS AND METHODS:

Collection of samples

Samples (Excreta of wild ass) were collected from little rann of Kutch, from wild ass sanctuary near Dhrangadhra, Gujarat, India [Latitude- 22°98'4.181"N and Longitude- 71°51'0.242"E].

Enrichment and isolation of halophiles

Halophiles were enriched in halophilic broth (Himedia) containing Casein acid hydrolysate-10 (g/l), Yeast extract- 10 (g/l), Protease peptone-5 (g/l), Trisodium citrate- 3 (g/l), Potassium chloride- 2 (g/l), Magnesium sulfate- 25 (g/l), Sodium chloride- 50-150 (g/l), pH- 7.0-7.4 as well as complete media broth containing Glucose- 10 (g/l), Potassium dihydrogen phosphate- 10 (g/l), Yeast extract- 5 (g/l), Peptone- 5 (g/l), Sodium chloride- 50-150 (g/l), pH- 7.0-7.4. From enriched 15% NaCl (w/v) halophilic broth and complete media broth organisms were streaked on respective agar media by four sector method for the purpose of isolation into pure culture. Total 24 isolates were obtained, designated as Mk-1 to Mk-24 and preserved on N-agar slant at 4˚C for further studies.

Enzyme assay

Lipase activity was determined as described by Pignede et al., [11].The substrate emulsion was prepared with, 50 ml. olive oil. The reaction mixture contained 1 ml enzyme, 5 ml substrate and 2 ml of 50mM phosphate buffer, pH 6.8 and was incubated for 1 hour at 37ºc with shaking. The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1µmole of fatty acid per min.

Purification of lipase

The crude lipase was fractioned by adding ammonium sulphate. Various fractions were collected at 4˚C and redissolved in phosphate buffer (pH 7) followed by

dialysis by membrane (Himedia). Protein content was determined by Folin's method.

Characterization of lipase

1. Temperature optima of lipase

Temperature optimum was determined by incubating reaction mixture at 20˚C to 70˚C. Lipase activity was determined by the Pignede method as described earlier.

2. Thermal stability

Thermal stability of lipase was determined by incubating enzyme at 60˚C and 70˚C for 30 min, 1 hour and 2 hour followed by rapid cooling at 4˚C. Enzyme activity was measured and residual activity was calculated on the basis of available data.

3. Effect of pH on lipase

The effect of pH on lipase was determined by preparing the substrate emulsion in various buffers with varying pH. Buffers were Citrate phosphate buffer (pH 4-5); phosphate buffer (pH 6-7); Tris-HCl buffer (pH 8-9); Glycine-NaOH buffer (pH10). After incubation of reaction mixture for 1 hour in shaking condition, lipase activity was determined.

4. Effect of inorganic salts on lipase

Effect of inorganic salts on lipase activity was determined by incubating enzyme at 30˚C for 1 hour followed by determination of enzyme activity. Inorganic salts were NaCl (10 Mm), $BaCl_2$ (0.001 M), $MgCl_2$ (0.001 M), KCl (2 Mm), $FeSo_4$ (0.001 M), $CaCl_2$ (0.001 M), NaF (2 Mm), $MnCl_2$ (2 Mm), Ethylene diamine tetra acetic acid (0.5%), Sodium dodecyl sulphate (0.5%). To determine effect of inorganic salts on lipase activity, it was compared with control containing no inorganic salts.

5. Effect of urea on lipase denaturation

Urea was used as denaturant at 8 M. The partially purified lipase was incubated for 1 hour and 2 hour. Residual activity was calculated by comparison with control containing no urea.

6. UV mutagenesis

All the three isolates were exposed to UV radiation in order to improve lipase secretion. Mutated cultures were compared with non-mutated cultures and enzyme activities were compared.

RESULTS AND DISCUSSION:

Pure cultures of 24 moderate halophiles isolated from wild ass excreta, designated as Mk-1 to Mk-24, were characterized for lipase secretion on solid media. On the basis of Zone ratio, three isolates were grown on liquid media and growth kinetics was studied.

Partial purification

Partial purification of lipase by ammonium sulfate fractionation gives different purification fold and % yield. Maximum purification fold was 3.44 and 70% yield from Mk-18 (Table-1). Above data are similar to lipase from *Fusarium oxysporum* [12]. Lipase from Mk-4 and Mk-23 gives much lower purification and yield as compare to Mk-18 and *Fusarium oxysporum.*

Characterization of lipase:

1. pH optima

pH is important factor that affect activity and stability of lipase. Lipase from Mk-4 and Mk-18 was active maximally at pH 5 while lipase from Mk-23 was showing maximum activity at pH 5-6 (Figure-1). Above data are incompatible with lipase from halotolerant *Staphylococcus warneri* PB233 [13], active at pH 7-8.

2. Temperature optima

Temperature is an environmental factor which affects protein folding and higher temperature generally denature enzyme. Lipase from moderate halophiles was active at mesophilic temperature range (30-40˚C). Higher temperature causes loss in enzyme activity (Figure-2). Similar types of results were also obtained from *R. oryzae* [14].

3. Temperature stability

Temperature stability data of lipase from moderate halophile states that lipase lose its half of activity after 60 min. and became inactive after 120 min. (Figure-3, 4). Lipases from moderate halophiles were less thermotolerant, contradictory, lipase from *Salinivibrio* sp. strain SA-2, which retain 90% of its activity at 80 °C for 30 min [15].

4. Effect of chemicals

As shown in figure-5, chemicals other than NaCl decrease enzyme activity while EDTA and SDS inactivate enzymes. The results are highly compatible with lipase from Marine *Vibrio fischeri* [16].

5. Urea denaturation

In the presence of 8M urea, lipases loose half of its activity after 60 min and retain little activity after 120 min from Mk-4 and Mk-23, while lipase from Mk-18 looses its activity after 60 min. (Figure-6).

6. UV mutagenesis

Increase in lipase production up to 3.4 U/ml from Mk-23 after UV mutagenesis (Figure-7). The yield is much lower as compare to mutant *Rhizopus oligosporus* IIB-63 [17].

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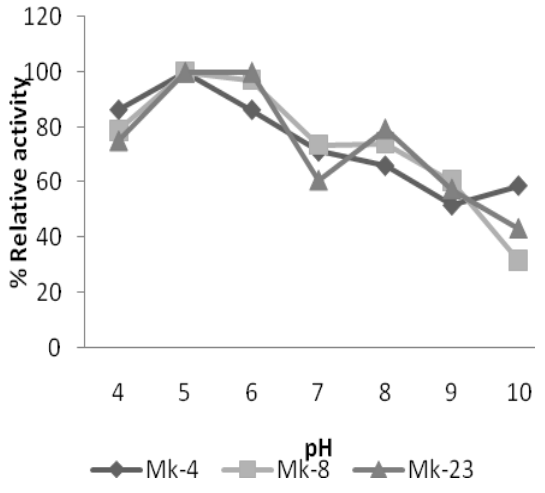
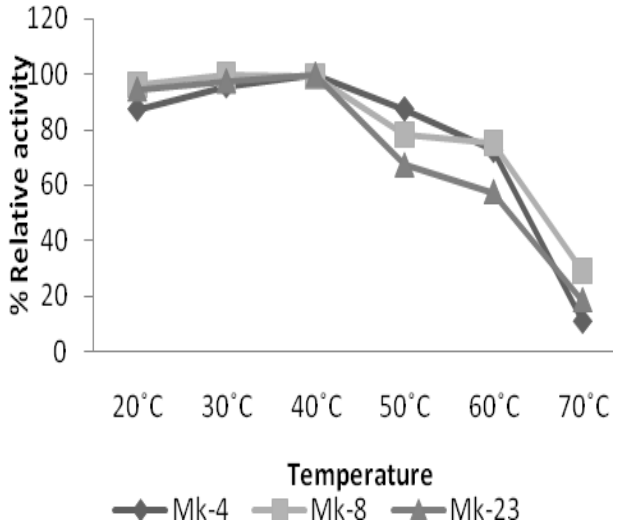
Table-1 Partial purification of lipase from moderate halophiles.

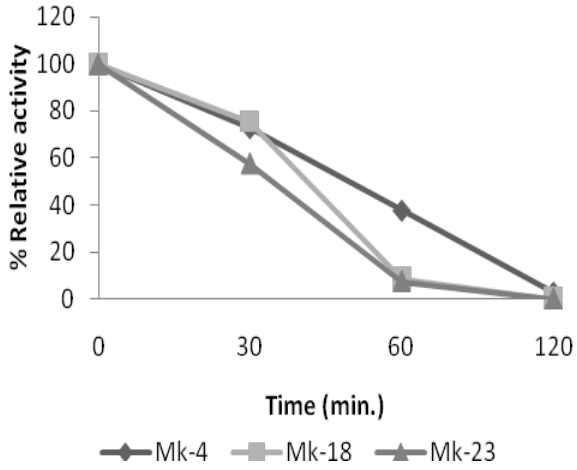
Isolate	Crude/Partially purified	Specific Activity (Units/mg)	Purification fold	Yield (%)
Mk-4	Crude	19.3	-	100
	Partially purified	26.41	1.36	33.3
Mk-18	Crude	11.6	-	100
	Partially purified	40	3.44	70
Mk-23	Crude	21.63	-	100
	Partially purified	42.72	1.98	42.2



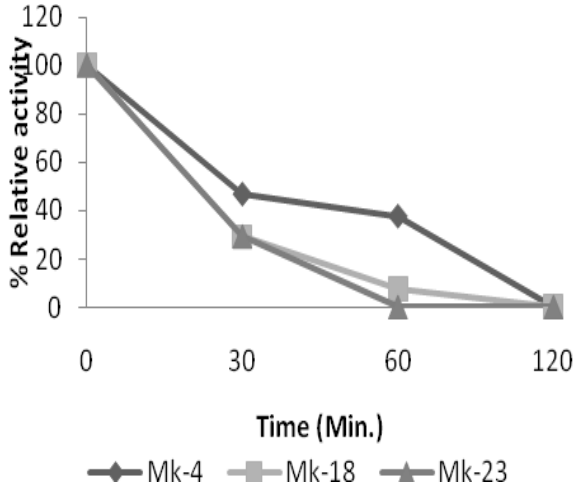
(Figure-1 pH optima of lipases)



(Figure-2 Temperature optima of lipases)



(Figure-3 Temperature stability of lipases at 60˚C)



(Figure-4 Temperature stability of lipases at 70˚C)

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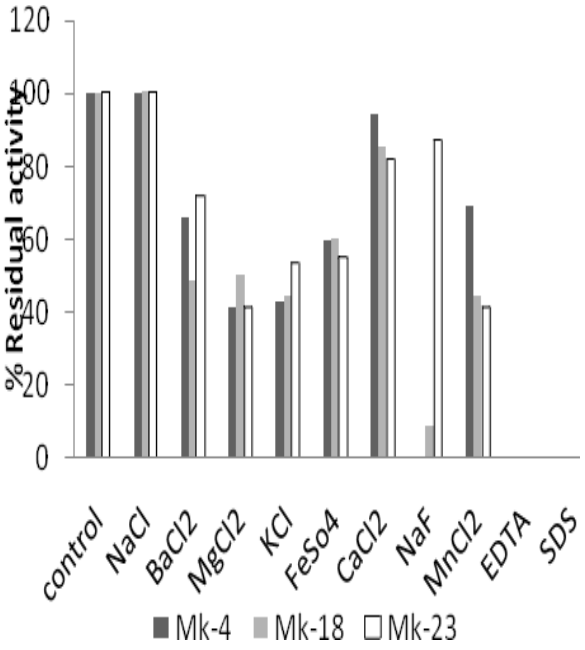
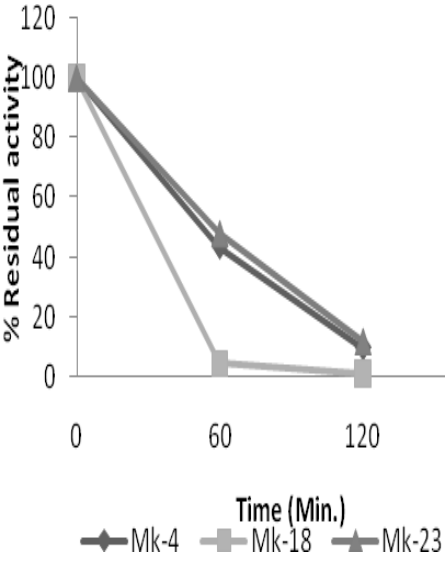
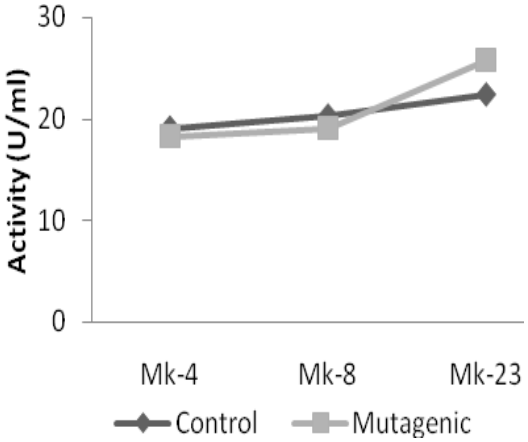


(Figure-5 Effect of various chemicals on lipase activity)



(Figure-6 Effect of 8M Urea on lipase activity)



(Figure-7 UV mutagenesis for strain improvement)

CONCLUSION:

Moderate halophilic bacterial community plays an important role in the production of salt and thermo-tolerant industrial enzymes. Lipases are the group of enzymes used in food, detergent, pharmaceutical, paper industries. Our aim is to explore comparative unexplored site i.e. wild ass intestinal microflora. Further, purification, characterization, strain improvement are necessary practices to cope up with efficient production of enzyme at industrial scale.

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